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**Brandt, SD, Kavanagh, PV, Westphal, F, Elliott, SP, Wallach, J, Colestock, T, Burrow, TE, Chapman, SJ, Stratford, A, Nichols, DE and Halberstadt, AL**

**Return of the lysergamides. Part II: Analytical and behavioural characterization of N6-allyl-6-norlysergic acid diethylamide (AL-LAD) and (2'S,4'S)-lysergic acid 2,4-dimethylazetidine (LSZ)**

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### Article

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**Return of the lysergamides. Part II: Analytical and behavioural characterization of *N*<sup>6</sup>-allyl-6-norlysergic acid diethylamide (AL-LAD) and (2'*S*,4'*S*)-lysergic acid 2,4-dimethylazetidide (LSZ)**

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Keywords:	New psychoactive substances, LSD, 5-HT <sub>2A</sub> receptors, Lysergamides, Psychedelics
Abstract:	<p>Lysergic N,N-diethylamide (LSD) is perhaps one of the most intriguing psychoactive substances known and numerous analogs have been explored to a varying extent in previous decades. In 2013, N<sup>6</sup>-allyl-6-norlysergic acid diethylamide (AL-LAD) and (2'<i>S</i>,4'<i>S</i>)-lysergic acid 2,4-dimethylazetidide (LSZ) have appeared on the 'research chemicals' / new psychoactive substances (NPS) market in both powdered and blotter form. This study reports the analytical characterization of powdered AL-LAD and LSZ tartrate samples and their semi-quantitative determination on blotter paper. Included in this study was the use of nuclear magnetic resonance spectroscopy, gas chromatography mass spectrometry (MS), low and high-resolution electrospray MS(/MS), high performance liquid chromatography diode array detection and GC solid-state infrared analysis. One feature shared by serotonergic psychedelics, such as LSD, is the ability to mediate behavioral responses via activation of 5-HT<sub>2A</sub> receptors. Both AL-LAD and</p>

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	<p>LSZ displayed LSD-like responses in male C57BL/6J mice when employing the head-twitch response (HTR) assay. AL-LAD and LSZ produced nearly identical inverted-U-shaped dose-dependent effects, with the maximal responses occurring at 200 µg/kg. Analysis of the dose-responses by nonlinear regression confirmed that LSZ (ED50 = 114.2 nmol/kg) was equipotent to LSD (ED50 = 132.8 nmol/kg) in mice, whereas AL-LAD was slightly less potent (ED50 = 174.9 nmol/kg). The extent to which a comparison in potency can be translated directly to humans requires further investigation. Availability of both chemical and pharmacological data obtained from NPS as they appear on the market provides important data to research communities that are interested in various aspects related substance use and forensic identification.</p>

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**Return of the lysergamides. Part II: Analytical and behavioural characterization of *N*<sup>6</sup>-allyl-6-norlysergic acid diethylamide (AL-LAD) and (2'S,4'S)-lysergic acid 2,4-dimethylazetidide (LSZ)**

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**Running title:** Analytical and behavioral characterization of (2'S,4'S)-LSZ and AL-LAD

**Keywords:** New psychoactive substances; LSD; 5-HT<sub>2A</sub> receptors; lysergamides; psychedelics

Abstract

Lysergic *N,N*-diethylamide (LSD) is perhaps one of the most intriguing psychoactive substances known and numerous analogs have been explored to a varying extent in previous decades. In 2013, *N*<sup>6</sup>-allyl-6-norlysergic acid diethylamide (AL-LAD) and (2'S,4'S)-lysergic acid 2,4-dimethylazetidide (LSZ) have appeared on the 'research chemicals' / new psychoactive substances (NPS) market in both powdered and blotter form. This study reports the analytical characterization of powdered AL-LAD and LSZ tartrate samples and their semi-quantitative determination on blotter paper. Included in this study was the use of nuclear magnetic resonance spectroscopy, gas chromatography mass spectrometry (MS), low and high-resolution electrospray MS(/MS), high performance liquid chromatography diode array detection and GC solid-state infrared analysis. One feature shared by serotonergic psychedelics, such as LSD, is the ability to mediate behavioral responses *via* activation of 5-HT<sub>2A</sub> receptors. Both AL-LAD and LSZ displayed LSD-like responses in male C57BL/6J mice when employing the head-twitch response (HTR) assay. AL-LAD and LSZ produced nearly identical inverted-U-shaped dose-dependent effects, with the maximal responses occurring at 200 µg/kg. Analysis of the dose-responses by nonlinear regression confirmed that LSZ (ED<sub>50</sub> = 114.2 nmol/kg) was equipotent to LSD (ED<sub>50</sub> = 132.8 nmol/kg) in mice, whereas AL-LAD was slightly less potent (ED<sub>50</sub> = 174.9 nmol/kg). The extent to which a comparison in potency can be translated directly to humans requires further investigation. Availability of both chemical and pharmacological data obtained from NPS as they appear on the market provides important data to research communities that are interested in various aspects related substance use and forensic identification.

Introduction

Lysergic *N,N*-diethylamide (LSD, lysergide) (Figure 1) is perhaps one of the most intriguing psychoactive substances known, given how strongly it has impacted scientific and cultural events. The psychoactive properties of LSD were discovered serendipitously by Dr. Albert Hofmann in 1943.<sup>[1-5]</sup> Subsequently, numerous structural modifications of the prototypical lysergamide scaffold have been explored,<sup>[6]</sup> forming the basis for investigations into the nature of the receptor binding and functional activity of LSD.<sup>[7-11]</sup> In some cases, information about the effects of several lysergamides in humans is available.<sup>[12]</sup>

Although not a new phenomenon,<sup>[13]</sup> the increasing rate at which 'research chemicals' or new psychoactive substances (NPS) have appeared on the market in recent years has attracted the attention of researchers and policy makers alike.<sup>[14-16]</sup> One LSD derivative, 1-propionyl-*d*-lysergic acid diethylamide (1P-LSD) (Figure 1), a substance that had never appeared in published literature, was recently encountered on the 'research chemical' market. Investigations carried out in the authors' laboratories characterized 1P-LSD using analytical and behavioral techniques.<sup>[17]</sup>

Two other lysergamides, (8β)-*N,N*-diethyl-6-(prop-2-en-1-yl)-9,10-didehydroergoline-8-carboxamide, also known as *N*<sup>6</sup>-allyl-6-norlysergic acid diethylamide and AL-LAD,

and [(2*S*,4*S*)-2,4-dimethylazetidin-1-yl][(8*β*)-6-methyl-9,10-didehydroergolin-8-yl]methanone, i.e. (2'*S*,4'*S*)-lysergic acid 2,4-dimethylazetide (LSZ, LA-SS-Az), appeared on the 'research chemicals' market in 2013, but have received little attention in the published literature. The first report of LSZ detection was received by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) in December 2013,<sup>[18]</sup> whereas detection of AL-LAD was reported for the first time in February 2015.<sup>[19]</sup>

AL-LAD is one of several lysergamides that have been modified at the *N*<sup>6</sup>-position.<sup>[7,12]</sup> The synthesis of AL-LAD was first described in 1976.<sup>[20]</sup> The substance was also synthesized by Hoffman and Nichols using a modified procedure published in 1985.<sup>[21]</sup> Like LSD, AL-LAD induces hyperthermia in rabbits.<sup>[22]</sup> When tested in the isolated rat uterus preparation, AL-LAD was found to have a more potent contractile effects than LSD.<sup>[23]</sup> The first investigation of the hallucinogen-like activity of AL-LAD was conducted by Hoffman and Nichols, who found that AL-LAD produced full substitution in rats trained to discriminate 0.08 mg/kg LSD tartrate.<sup>[21]</sup> Furthermore, the potency of AL-LAD in the drug discrimination paradigm ( $ED_{50}$  = 13 nmol/kg) exceeded that of LSD ( $ED_{50}$  = 46 nmol/kg).<sup>[21]</sup> Receptor binding studies revealed that AL-LAD had high affinity for 5-HT<sub>2A</sub> receptors labeled with [<sup>3</sup>H]ketanserin ( $K_i$  = 8.1 nM) and ([<sup>125</sup>I]-*R*-DOI ( $K_i$  = 3.4 nM) in rat frontal cortex homogenates.<sup>[8,24]</sup> Subsequently, it was shown that AL-LAD displayed slightly lower affinity than LSD for D<sub>1</sub> ( $K_{0.5}$  = 189 nM vs. [<sup>3</sup>H]SCH-23390) and D<sub>2</sub> ( $K_{0.5}$  12.3 nM vs. [<sup>3</sup>H]spiperone in the presence of ketanserin) dopaminergic receptors.<sup>[25]</sup>

The isomers of lysergic acid 2,4-dimethylazetide (LSZ) were prepared to gain further insight into the binding orientation of LSD and to help map the topography of the 5-HT<sub>2A</sub> receptor. Specifically, the 2,4-dimethylazetide analog of LSD was pursued as a sterically constrained version of the *N,N*-diethylamide group.<sup>[26]</sup> Because the presence of a 2,4-dimethylazetide moiety gives rise to two additional chiral centers, Nichols *et al.* synthesized three isomers of LSZ: *cis*-(*meso*)-, *trans*-(2'*R*,4'*R*)-, and *trans*-(2'*S*,4'*S*)-LSZ. Drug discrimination studies confirmed that all three isomers produce full substitution in LSD-trained rats and showed that the (2'*S*,4'*S*) isomer ( $ED_{50}$  = 25 nmol/kg) was more potent than LSD, whereas the other two isomers were less potent ( $ED_{50}$  *cis* = 115 nmol/kg;  $ED_{50}$  (2'*R*,4'*R*) = 134 nmol/kg) and only the (2'*S*,4'*S*) isomer fully substituted in rats trained to discriminate the 5-HT<sub>1A</sub> agonist LY-293284. Receptor binding studies showed that of the three LSZ isomers, the (2'*S*,4'*S*) isomer had activity most similar to LSD at a range of monoamine receptors.<sup>[26,27]</sup> An emerging body of evidence suggests that 5-HT<sub>2A</sub>-mediated agonist effects also may play an important role in the manifestation of anti-inflammatory responses.<sup>[5]</sup> The first indications for this came from investigations with the classical serotonergic psychedelic (*R*)-2,5-dimethoxy-4-iodoamphetamine (DOI), which was found to suppress tumor necrosis factor (TNF)- $\alpha$ -induced inflammation processes in a number of tissues, for example by inhibition of pro-inflammatory gene expression.<sup>[28]</sup> Interestingly, (2'*S*,4'*S*)-LSZ was also able to induce a TNF- $\alpha$ -mediated increase in pro-inflammatory gene expression at concentrations of 1 and 50 nM,<sup>[28,29]</sup> consistent with its ability to function as a 5-HT<sub>2A</sub> receptor agonist. These particular properties may have been the reason why (2'*S*,4'*S*)-LSZ was included in more

recently published patent literature associated with hyposmia (smell loss) and the treatment of viral infections.<sup>[30,31]</sup>

Although available on the 'research chemical' market for some time, a comprehensive analytical characterization has not yet been described in detail for either AL-LAD or LSZ. Some data, however, have been published for AL-LAD, including the analysis of both the 8 $\alpha$  and 8 $\beta$  epimers (iso-AL-LAD and AL-LAD, respectively) by nuclear magnetic resonance spectroscopy (NMR), the implementation of high performance liquid chromatography (HPLC) triple quadrupole tandem mass spectrometry (MS/MS) used for the generation of tandem mass spectra,<sup>[32]</sup> and a chemiluminescent nitrogen detection method coupled to HPLC.<sup>[33]</sup>

The present investigation aimed to fill this knowledge gap by performing an extensive characterization of powdered AL-LAD and LSZ samples and commercially obtained blotter paper samples (photographs supplied as Supporting Information). Included in this approach was the use of NMR, gas chromatography mass spectrometry (GC-MS), high-resolution electrospray MS/MS, HPLC diode array detection, HPLC quadrupole MS, and GC solid-state infrared analysis. A semi-quantitative analysis of AL-LAD and LSZ blotters was also incorporated. 5-HT<sub>2A</sub> receptor activation plays a central role in the mediation of psychedelic effects in humans.<sup>[3,5]</sup> Likewise, 5-HT<sub>2A</sub> activation is responsible for the head-twitch response (HTR), a behaviour often used to assess hallucinogen-like effects in rodents.<sup>[34,35]</sup> Because it was predicted that both AL-LAD and LSZ would induce the HTR, a test of this behavioral response was included in this investigation using a quantitative, magnetometer-based approach.<sup>[35]</sup>

## Experimental

### Materials

All chemicals used were of analytical or HPLC grade and were obtained either from Aldrich (Dorset, UK), Rathburn Chemicals Ltd (Walkerburn, Scotland, UK) or Fisher Scientific (Dublin, Ireland). Powdered samples of (2'S,4'S)-lysergic acid 2,4-dimethylazetidine (LSZ) and *N*<sup>6</sup>-allyl-6-norlysergic acid diethylamide (AL-LAD) tartrate were obtained from Synex Ltd. (London, UK). Blotters labeled to contain 150  $\mu$ g AL-LAD and 150  $\mu$ g LSZ, and blank blotter paper, were provided by the same vendor. LSD was used as an internal standard (stock solution at 1 mg/mL) and purchased from Cerilliant (Round Rock, TX, USA). A synthesized reference sample of (2'S,4'S)-LSZ hemitartrate was available from work published previously.<sup>[26]</sup>

### Instrumentation

#### *Nuclear magnetic resonance spectroscopy*

NMR spectra were recorded in d<sub>6</sub>-DMSO (Sigma-Aldrich, St. Louis, MO, USA) using an Agilent DD2-700 spectrometer with an Agilent HFCN cryogenically cooled 5-mm probe (<sup>1</sup>H at 699.81 MHz; <sup>13</sup>C at 175.98 MHz) (Agilent, Santa Clara, CA, USA). Proton spectra were acquired using 64 scans with a 45° pulse (3.6  $\mu$ s) and a 5.0



s relaxation delay, collecting 50223 points, with an acquisition time of 4.5 s and spectral window of 11160 Hz. Carbon spectra were acquired using 4096 scans with a 30° pulse (3.6  $\mu$ s), WALTZ-16 decoupling, and a 0.2 s relaxation delay, collecting 64k data points, with an acquisition time of 1.57 s and spectral window of 41667 Hz. Suggested assignments were aided by 1-D and 2-D experiments. Internal chemical shift references were based on residual solvent peaks.

#### *Gas chromatography mass spectrometry*

Electron ionization (EI) mass spectra (70 eV) were recorded using a Finnigan TSQ 7000 triple stage quadrupole mass spectrometer coupled to a gas chromatograph (Trace GC Ultra, Thermo Electron, Dreieich, Germany). Sample introduction was carried out using a CTC CombiPAL (CTC Analytics, Zwingen, Switzerland) autosampler. The emission current was 200  $\mu$ A and the scan time was 1 s spanning a scan range between  $m/z$  29 –  $m/z$  600. The ion source temperature was maintained at 175 °C. Samples were introduced via gas chromatography with splitless injection using a fused silica capillary DB-1 column (30 m  $\times$  0.25 mm, film thickness 0.25  $\mu$ m). The temperature program consisted of an initial temperature of 80 °C, held for 1 min, followed by a ramp to 280 °C at 15 °C/min. The final temperature was held for 21 min. The injector temperature was 220 °C. The transfer line temperature was maintained at 280 °C and the carrier gas was helium in constant flow mode at a flow rate of 1.0 mL/min. Approximately 2 mg were dissolved in 1.5 mL methanol. For analysis, 1  $\mu$ L sample solution was injected into the GC-MS system. Retention indices (RI) are given as Kovats Indices calculated from measurement of a *n*-alkane mixture analyzed with the above mentioned temperature program.

#### *High-resolution electrospray mass spectrometry*

Ultrahigh-performance liquid chromatography quadrupole time-of-flight single and tandem mass spectrometry (UHPLC-QTOF-MS/MS) data were recorded on an Agilent 6540 UHD Accurate-Mass Q-TOF LC-MS system coupled to an Agilent 1290 Infinity UHPLC system (Agilent, Cheshire, UK). Chromatographic separation was achieved using an Agilent Zorbax Eclipse Plus C18 column (100 mm  $\times$  2.1 mm, 1.8  $\mu$ m) (Agilent, Cheshire, UK). Mobile phases used for UHPLC separation consisted of 100% acetonitrile (1% formic acid) and an aqueous solution of 1% formic acid. The column temperature was set at 40 °C (0.6 mL/min) and data were acquired for 5.5 min. The elution was a 5–70% acetonitrile gradient ramp over 3.5 min, then increased to 95% acetonitrile in 1 min and held for 0.5 min before returning to 5% acetonitrile in 0.5 min. QTOF-MS data were acquired in positive mode scanning from  $m/z$  100 –  $m/z$  1000 with and without auto MS/MS fragmentation. Ionization was achieved with an Agilent JetStream electrospray source and infused internal reference masses. Agilent 6540 QTOF-MS parameters: gas temperature 325 °C, drying gas 10 L/min and sheath gas temperature 400 °C. Internal reference ions at  $m/z$  121.05087 and  $m/z$  922.00979 were used for calibration purposes.

#### *Liquid chromatography diode array detection*

HPLC-DAD was carried out on a Dionex 3000 Ultimate liquid chromatography system coupled to a UV diode array detector (Thermo Fisher, St. Albans, UK), using a Phenomenex Synergi Fusion column (150 mm × 2mm, 4 µm) protected by a 4 mm × 3 mm Phenomenex Synergi Fusion guard column (Phenomenex, Cheshire, UK). Mobile phases used were 70% acetonitrile with 25 mM of triethylammonium phosphate buffer (TEAP) and aqueous solution of 25 mM TEAP buffer. A gradient elution started from 4% acetonitrile and ramped to 70% acetonitrile in 15 min and held for 3 min, with total acquisition time of 18 min at a flow rate of 0.6 mL/min. The diode array detection window was set at 200 nm – 595 nm (collection rate 2 Hz).

#### *Liquid chromatography electrospray mass spectrometry*

LC-MS analyses were performed on an Agilent 1100 system. Separation was obtained on a Restek (Bellefonte, PA, USA) Allure PFPP column (5 µm, 50 × 2.1 mm). Mobile phase A consisted of 0.1% formic acid in water, whereas, mobile phase B consisted of 0.1% formic acid in acetonitrile. The Agilent LC-MSD settings were as follows: positive electrospray mode, capillary voltage 3500 V, drying gas (N<sub>2</sub>) 12 L/min at 350 °C, nebulizer gas (N<sub>2</sub>) pressure 50 psi, Scan mode *m/z* 70 – 500, fragmentor voltage 150 V. The sample for LC-MS analysis was dissolved in acetonitrile/water (1:1, containing 0.1% formic acid) at a concentration of 10 µg/mL. The injection volume was 1 µL, flow rate was 0.80 mL/min and the column temperature was 30 °C. The total run time was 25 min. The following gradient elution program was used: 0–2 min 2% B, followed by an increase to 60% within 15 min, then up to 80% within 20 min, returning to 2% within 25 min.

#### *Gas chromatography solid-state infrared analysis*

The methanolic solution was measured on a GC-solid phase-IR-system consisting of an Agilent GC 7890B (Waldbronn, Germany) with probe sampler Agilent G4567A and a DiscovIR-GC™ (Spectra Analysis, Marlborough, MA, USA). The column eluent was cryogenically accumulated on a spirally rotating ZnSe disk that was cooled by liquid nitrogen. The IR spectra were directly recorded through the IR-transparent ZnSe disk using a nitrogen-cooled MCT detector. GC parameters: the injection was carried out in splitless mode with an injection port temperature of 240 °C and a DB-1 fused silica capillary column (30 m × 0.32 mm i.d., 0.25 µm film thickness). The carrier gas was helium with a flow rate of 2.5 mL/min; oven temperature program: 80 °C for 2 min, ramped to 290 °C at 20 °C/min, and held at the final temperature for 25 min. The transfer line heater was set at 280 °C. Infrared conditions: oven temperature, restrictor temperature, disc temperature, and Dewar cap temperatures were 280 °C, 280 °C, –40 °C, and 35 °C, respectively. The vacuum was 0.2 mTorr, disc speed 3 mm/s, spiral separation was 1 mm, wavelength resolution 4 cm<sup>-1</sup> and IR range 650–4000 cm<sup>-1</sup>. Acquisition time was 6s/file with 64 scans/spectrum. Data were processed using GRAMS/AI Ver. 9.1 (Grams Spectroscopy Software Suite, Thermo Fischer Scientific) followed by implementation of the OMNIC Software, Ver. 7.4.127 (Thermo Electron Corporation, Dreieich, Germany).

#### *Liquid chromatography ultraviolet detection used for analysis of blotters*

HPLC analyses were performed on a Hitachi LaChrom Elite system that comprised an L-2130 pump, L-2200 autosampler, L-2300 column oven, and L-2400 UV detector, respectively (Hitachi High Technologies America, Inc., San Jose, CA, USA). The detection wavelength was set at 250 nm. Separation was obtained using a Zorbax Eclipse Plus C18 column (5  $\mu$ m, 250 mm  $\times$  4.6 mm) from Agilent Technologies (Santa Clara, CA, USA). Mobile phase A consisted of methanol with 0.1% formic acid (v/v) and mobile phase B was aqueous 10 mM ammonium formate titrated to pH 3.3. The following gradient was used for elution: 0–2 min 28% B, followed by an increase to 43% within 22 min and held for 6 min. This was followed by a decrease to 28% B within 3 min and held for 7 min to give a total run time of 40 min. Chromatograms were analyzed using the Agilent OpenLAB CDS EZChrom Edition (Agilent Technologies, Santa Clara, CA, USA).

#### *Analyses of blotter samples*

A sample buffer containing LSD as internal standard (LSD buffer) was prepared by diluting a 1 mg/mL stock solution to a final concentration of 0.025 mg/mL using a 50:50 mixture of mobile phase A and B. Powdered LSZ and AL-LAD were dissolved in LSD buffer to give a stock concentration of 1 mg/mL, and then diluted to desired concentration levels at 250, 200, 150, 100, 50, and 10  $\mu$ g/mL, respectively. The injection volume of samples was 10  $\mu$ L, flow rate was 0.6 mL/min, and the column temperature was set at 35  $^{\circ}$ C using an external column oven from Cole-Parmer (Illinois, USA) to account for the column length of 25 cm.

Blank blotter paper was spiked with 100  $\mu$ L of a solution of 100  $\mu$ g LSZ or AL-LAD per mL of methanol and dried at room temperature protected from light. One hundred  $\mu$ L of a 100  $\mu$ g/mL solution were added separately to an amber vial and dried in a desiccator to serve as a control sample. Dried blotters were transferred to amber vials and extracted three times with LSD buffer (333, 333, and 334  $\mu$ L) using an ultrasonic bath (Branson 1800, Danbury, CT, USA) at room temperature. The combined extracts were analyzed without further dilution by HPLC and compared to the control samples to determine recovery from the spiked blotter paper. LSZ and AL-LAD blotters were analyzed in the same manner as described for the control blotters.

#### **Head-twitch response assays**

Male C57BL/6J mice (6–8 weeks old) were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and housed up to four per cage with a reversed light-cycle (lights on at 1900 h, off at 0700 h). Food and water were provided *ad libitum*, except during behavioral testing, which was performed between 1000 h and 1830 h. The head twitch response (HTR) was detected using a head mounted magnet and a magnetometer coil, as described previously.<sup>[35]</sup> Behaviour was monitored for 30 min immediately after IP injection of vehicle (saline) or drug. The injection volume was 5 mL/kg. HTR counts were analyzed by one-way ANOVA and *post-hoc* comparisons were made using Tukey's studentized range method. Significance was demonstrated by surpassing an  $\alpha$ -level of 0.05. ED<sub>50</sub> values and 95% confidence limits were calculated using nonlinear regression.

## Results and discussion

### Analytical characterization

Electron ionization (EI) mass spectra for AL-LAD and LSZ are shown in Figures 2 and 3B–D. The molecular ions for both compounds displayed a high relative abundance ( $m/z$  349 for AL-LAD and  $m/z$  335 for LSZ) that have also been observed for other lysergamides such as 1P-LSD and LSD.<sup>[17]</sup> Suggested fragmentation pathways have been added as Supporting Information and follow on from those previously described and discussed in detail for 1P-LSD and LSD.<sup>[17]</sup> The structural similarity between AL-LAD and LSD (Figure 1) revealed the presence of similar fragment ions (Supporting Information). Examples include the iminium ion at  $m/z$  72 or the retro Diels-Alder species at  $m/z$  280 (Figure 2, Supporting Information). The loss of an allyl radical from the nitrogen at the 6-position was represented by the formation of a prominent fragment at  $m/z$  308. In the spectrum of LSZ, the presence of the azetidine group shifted the retro Diels-Alder fragment to  $m/z$  292 (Figure 3D). Other similarities observed in the mass spectra between LSD and AL-LAD included fragment clusters between  $m/z$  151 –  $m/z$  154 and  $m/z$  178 –  $m/z$  182 and these have also been observed in the EI mass spectra of 1P-LSD, LSD, and LSZ (Figure 3B–3D, Supporting Information).<sup>[17]</sup> AL-LAD product ions have been reported previously following triple quadrupole MS/MS analysis. Some of the product ions detected in the present study were in agreement although the  $m/z$  309 detected here was not reported previously.<sup>[32]</sup>

An interesting observation was made during GC-MS analysis of LSZ. As shown in Figure 3A, three major, distinct peaks were detected and the corresponding EI mass spectra are shown in Figure 3B–3D. At first glance, the similarity of these mass spectra suggested the detection of three LSZ isomers and a closer inspection revealed that some differences were also noticeable, especially in the mass range  $m/z$  223 –  $m/z$  337. Interestingly, only the mass spectrum of LSZ-III (Figure 3D) showed the retro Diels-Alder fragment at  $m/z$  292. It seemed conceivable that these additional isomers might have been formed as a consequence of GC-induced conditions (e.g. heat and/or active sites) that might have led to this isomerization phenomenon. Furthermore, the mass spectra of two additional peaks of comparatively minor abundance (Figure 3A) at 26.29 and 26.72 min have also been included as Supporting Information. It has to be noted that the minor differences in retention times mentioned here and those shown on Figure 3A reflected a minor difference in peak picking. In total, five isomeric mass spectra have been detected but it was not possible to identify the exact configuration. As shown below, HPLC single quadrupole MS analysis of the powdered LSZ material revealed the presence of a diastereomer when implementing analysis of the protonated molecule at  $m/z$  350 in single ion monitoring mode. Whether this minor peak reflected the presence of the 8 $\alpha$ -epimer (iso-LSZ) could not be confirmed although it appeared to be a likely candidate.

Ultra high-performance liquid chromatography electrospray ionization accurate mass quadrupole time-of-flight tandem mass spectrometry (UHPLC-ESI-QTOF-MS/MS) data for AL-LAD are displayed in Figure 4, with suggested dissociation pathways as Supporting Information. The two most abundant product ions observed under QTOF-MS/MS conditions were  $m/z$  309.18410 and  $m/z$  208.09945, respectively. In the case of the former, the loss of the allyl group from the protonated molecule ( $C_{22}H_{28}N_3O^+$ ) would have been consistent with  $C_{19}H_{23}N_3O^{++}$  (309.18356,  $\Delta = 1.75$  ppm), whereas the latter might have been consistent with  $C_{14}H_{12}N_2^{++}$  (208.09950,  $\Delta = -0.24$  ppm). As suggested in the Supporting Information, the  $m/z$  208 product ion might have formed due to the loss of an allyl radical from an intermediate species at  $m/z$  249.13837 ( $C_{17}H_{17}N_2^+$ , 249.13862,  $\Delta = -1.00$  ppm). Figure 4B and 4C display the mass spectra obtained from analysis by HPLC-ESI single quadrupole mass spectrometry (LC-Q-MS) at two different fragmentor voltages. At these settings, sufficient in-source collision-induced dissociation (CID) allowed for the detection of product ions, most notably at  $m/z$  309, 281 and 208 (Figure 4B). The sodiated adduct ions were also present at  $m/z$  372. The analysis of powdered AL-LAD material by LC-Q-MS in selected ion monitoring mode (SIM) revealed only one visible peak (Figure 4D), suggesting the absence of a diastereomer, which was somewhat surprising because the powdered material showed significant discoloration (Supporting Information). The UV spectrum recorded by HPLC diode array detection (DAD) is presented in Figure 4E and is consistent with the spectrum recorded for LSD (Supporting Information).

UHPLC-ESI-QTOF-MS/MS, LC-Q-MS and LC-DAD data collected from LSZ are summarized in Figure 5, with suggested dissociation pathways in the Supporting Information section. As was the case with AL-LAD, the LSZ-derived counterpart of the  $m/z$  281 ion in the AL-LAD spectrum was observed at  $m/z$  293.16470 ( $C_{19}H_{21}N_2O^+$ , 293.16484,  $\Delta = -0.48$  ppm). Similarly, loss of 2,4-dimethylazetidine-1-carbaldehyde might have given rise to  $m/z$  223.12345 ( $C_{15}H_{15}N_2^+$ , 223.12297,  $\Delta = 2.15$  ppm). The  $m/z$  223 ion might have also been formed via the formation of an oxonium intermediate ( $C_{16}H_{15}N_2O^+$ , 251.11789,  $\Delta = -0.40$  ppm), followed by a neutral loss of CO (Supporting Information). The corresponding oxonium intermediate in the AL-LAD spectrum would have been expected at  $m/z$  277.13354 ( $C_{18}H_{17}N_2O^+$ ) before yielding the  $m/z$  249 ion, but the  $m/z$  277 species was not observed in any appreciable abundance. The LC-Q-MS investigation in SIM mode revealed the presence of a second peak at 11.52 min (Figure 5F), labeled as LSZ-2, which indicated the potential presence of a diastereomer, for example iso-LSZ, although that could not be confirmed due to the lack of authentic reference material. The LC-DAD spectrum recorded for LSZ was comparable to the spectrum of AL-LAD (Figure 4E) and LSD (Supporting Information).

Following analysis of both lysergamides by GC-MS, the solid-state infrared (IR) spectra of the eluents were recorded when cryogenically deposited on a spirally rotating cryogenically cooled zinc selenide disk. The advantage of this approach was that the solidified free base material was measured, which circumvented a problem encountered with several other lysergamides where mixtures of salt forms can occur due to changes during storage and polymorphism.<sup>[36]</sup> The ATR-FTIR spectrum of AL-LAD is shown in Figure 6. Key features also observed for LSD, such as the C=O and C-N stretch or C-H out-of-plane deformations suggested to be associated with the



indole ring, have also been observed.<sup>[36-39]</sup> The NH bands at higher wavenumbers were present as expected but this was not observed previously in the spectrum recorded for 1P-LSD under identical conditions due to the attachment of the propionyl group.<sup>[17]</sup> As described above, GC-MS analysis of LSZ revealed the detection of what appeared to be LSZ isomers. The three most predominant peaks LSZ-I – LSZ-III (Figure 3A) not only displayed some differences in the mass spectra, but inspection of the corresponding IR spectra also uncovered some minor differences. Figure 7 shows partial, overlaid spectra of LSZ isomers I – III to facilitate the comparison. All complete spectra can be found as Supporting Information.

The LSZ, both in powdered and blotter form and obtained as a 'research chemical', was manufactured and sold as the *trans*-(2'S,4'S)-isomer, which was consistent with results acquired by using a chiral HPLC method (Supporting Information). The comparison with the synthesized (2'S,4'S)-isomer<sup>[26]</sup> revealed the same retention time, which suggested that the 'research chemical' products were consistent with the information provided on the product label. However, without the comparison with the (2'R,4'R)-form, the possibility of co-elution could not be fully excluded. The amounts of AL-LAD and LSZ extracted from blotters were estimated based on the use of the available powdered material as the standard for calibration purposes at different concentration levels. However, LSZ was available as a synthesized standard. When employing this HPLC method, retention times observed for LSD (I.S.), LSZ, and AL-LAD were 28.39, 29.33, and 32.15 min, respectively (Supporting Information). Table 1 provides a summary of extracted amounts of AL-LAD and LSZ from blotters that were both labeled to contain 150 µg. Estimations of recovery were based on the extraction of identical blank blotter papers that had been used for the manufactured blotters. Recovery values obtained from spiked blank blotters were 91.5% (AL-LAD) and 93.3% (LSZ), respectively. The amounts of AL-LAD and LSZ on the commercially prepared blotters were estimated based on the recovery rates (Table 1). As mentioned below, the proton NMR data recorded for LSZ revealed that the integrals for tartaric acid (~1.6H instead of either 1H or 2H) had to be taken into account, thus, leading to LSZ results to be expressed as freebase equivalents. AL-LAD results, on the other hand, were expressed based on the hemitartrate salt in alignment with NMR measurements. Since certified reference material was not available, the results obtained were considered semi-quantitative. The purity of AL-LAD powder was not known (see HPLC trace in Supporting Information) and since this material was used for constructing the calibration curve, the results obtained from this determination were also considered tentative.

Chemical shifts recorded by nuclear magnetic resonance spectroscopy (NMR) were assigned using chemical shift position, multiplicities, as well as 2-D spectra including HQSC and COSY.  $\alpha$  and  $\beta$   $^1\text{H}$  chemical shifts were assigned using the approach described previously.<sup>[17]</sup> The chemical shift positions and multiplicities of the  $^1\text{H}$  NMR spectra recorded for AL-LAD were compared with previous reports published by Hoffman and Nichols<sup>[21]</sup> and Stachulski *et al.*<sup>[40]</sup> Slight differences in chemical shift position was likely due to the use of  $\text{CDCl}_3$  in the earlier study compared to  $\text{d}_6$ -DMSO used here. The current assignments, provided in Table 2, expand upon earlier reports in which a number of the chemical shifts were not assigned.  $^{13}\text{C}$  NMR assignments for AL-LAD did not appear to have been available in the reported

literature. The C-22 carbon gave separate  $^{13}\text{C}$  chemical shifts at 41.50 and 39.48 ppm, which was due to non-equivalence of these methylene carbons and hindered rotation about the amide C-N bond. The  $^{13}\text{C}$  NMR shift at 39.48 ppm was found to couple to a single quartet (2H) at 3.31 ppm, suggesting free rotation of this  $\text{CH}_2$  and coupling with the  $\text{CH}_3$  of H-23. In the case of the 41.50 ppm shift, the HSQC showed coupling to two doublet of quartets at 3.46 and 3.40 ppm. This splitting pattern is consistent with the fact that this is a prochiral site and the distinct chemical environment experienced by these protons relative to one another and the other C-22 protons. Thus, these doublets of quartets resulted from germinal coupling ( $J = 14.4$  Hz) and vicinal coupling ( $J = 7.2$  Hz) with the  $\text{CH}_3$  of H-23. This phenomenon was also observed with 1P-LSD.<sup>[17]</sup> Another point worth noting was the integration of the tartaric acid peak in the  $^1\text{H}$  spectra at 4.26 ppm. This shift integrated to 1 proton consistent with the hydrogen 2,3-dihydroxybutanoate (hemitartrate) salt of AL-LAD.

The  $^1\text{H}$  spectrum of LSZ was consistent with that published previously (hemitartrate in  $\text{d}_6$ -DMSO) by Nichols *et al.*<sup>[26]</sup> The NMR data ruled out the presence of the *cis*-form but differentiation between the two *trans* enantiomers was not possible.  $^1\text{H}$  chemical shift assignments of LSZ and  $^{13}\text{C}$  NMR data for LSZ have not been previously reported. Assignments were made in the same manner as AL-LAD and are provided in Table 3. In contrast to AL-LAD, the tartaric acid proton shift at 4.22 ppm integrated to ~1.6 protons. This integration was inconsistent with the hemi- or stoichiometric tartrate salts, giving a ratio of LSZ:tartaric acid of 1:0.8. This could be due to a mixed salt (hemi and stoichiometric) or an excess of free tartaric acid present as an impurity in the hemitartrate salt.<sup>[39]</sup> The  $^1\text{H}$  and  $^{13}\text{C}$  chemical shifts of the dimethylazetidine ring were as expected. In the  $^1\text{H}$  spectrum, the H-21 protons gave two separate multiplets at 4.66 – 4.59 ppm and 4.39 – 4.31 ppm. The two methyl groups (H-22) were assigned to two doublets at 1.44 and 1.35 ppm due to coupling ( $J = \sim 6.2$  Hz) with H-21. In contrast, *cis*-LSZ H-22 protons appeared (no assignments given) to give a single multiplet at 1.28 – 1.41 ppm integrating to 6H.<sup>[26]</sup> The H-23 protons of the dimethylazetidine ring were assigned to two separate doublets of doublets of doublets at 1.99 and 1.95 ppm from both geminal and vicinal couplings.

### Head-twitch response

The head twitch response (HTR) was assessed for 30 min following administration of AL-LAD and LSZ. Both lysergamides induced the HTR (AL-LAD:  $F_{5,26} = 20.45$ ,  $p < 0.0001$ ; LSZ:  $F_{5,25} = 15.52$ ,  $p < 0.0001$ ; Figure 8). As shown in Table 4, AL-LAD and LSZ produced nearly identical inverted-U-shaped dose-dependent effects, with the maximal responses occurring at 200  $\mu\text{g}/\text{kg}$ . The effects of LSD on HTR, published in an earlier study, are included in Table 4 for comparison. Analysis of the dose-responses by nonlinear regression confirmed that LSZ was equipotent to LSD in mice, whereas AL-LAD was slightly less potent (Table 4). The potency values (nmol/kg) determined for LSZ were corrected based on the proton NMR data (0.8 molar equivalent of tartaric acid relative to LSZ). The magnitude of the maximal response to AL-LAD, as far as the HTR counts were concerned (mean  $\pm$  SEM =  $53.0 \pm 2.4$ ), was greater than that of LSZ ( $33.6 \pm 2.7$ ). Normalizing the responses

(as %baseline) confirmed that AL-LAD induced a significantly larger response than LSZ ( $t_9 = 7.02$ ,  $p < 0.0001$ ).

1P-LSD has also been shown to induce the HTR.<sup>[17]</sup> In contrast to AL-LAD and LSZ, however, 1P-LSD was found to be significantly less potent than LSD. There is some evidence that the maximum number of head twitches induced by a compound in rats may reflect its efficacy at the 5-HT<sub>2A</sub> receptor.<sup>[41]</sup> To the extent that such a relationship exists in mice, the present results indicate that AL-LAD and especially LSZ may activate 5-HT<sub>2A</sub> receptors with lower relative efficacy compared with LSD. Alternatively, lysergamides exhibit activity at other receptors, including 5-HT<sub>1A</sub> and 5-HT<sub>2C</sub>, which may attenuate expression of the HTR.

## Conclusion

Both AL-LAD and LSZ were confirmed to induce LSD-like behavioural effects in mice, which added a multidisciplinary layer to the analytical characterization work described in the present study. The provision of a diverse set of chemical and pharmacological data obtained from new psychoactive substances as they appear on the market provides important data to research communities that are focused on various aspects of substance use and forensic identification. Further studies seem warranted to clarify the psychopharmacological properties of these substances.

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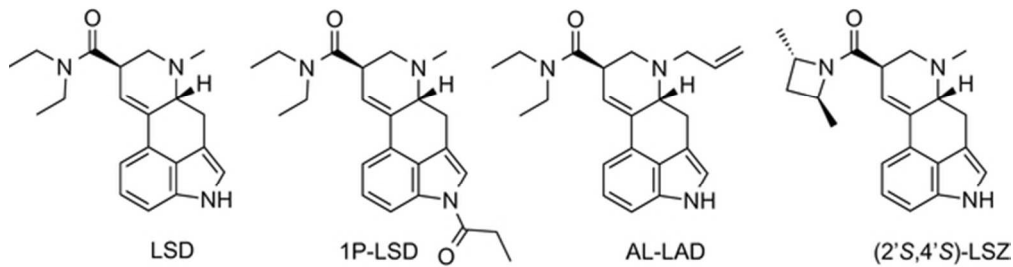


Figure 1. Structural representations of AL-LAD, LSZ and related lysergamides.  
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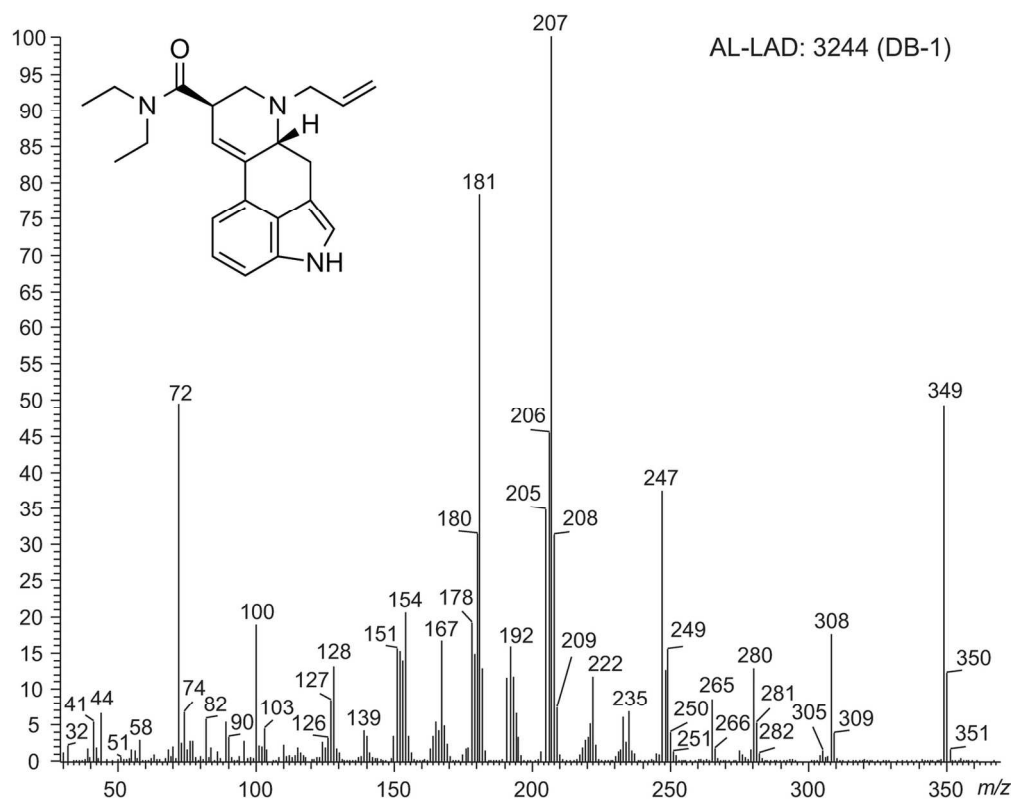


Figure 2. Electron ionization mass spectrum recorded for AL-LAD.  
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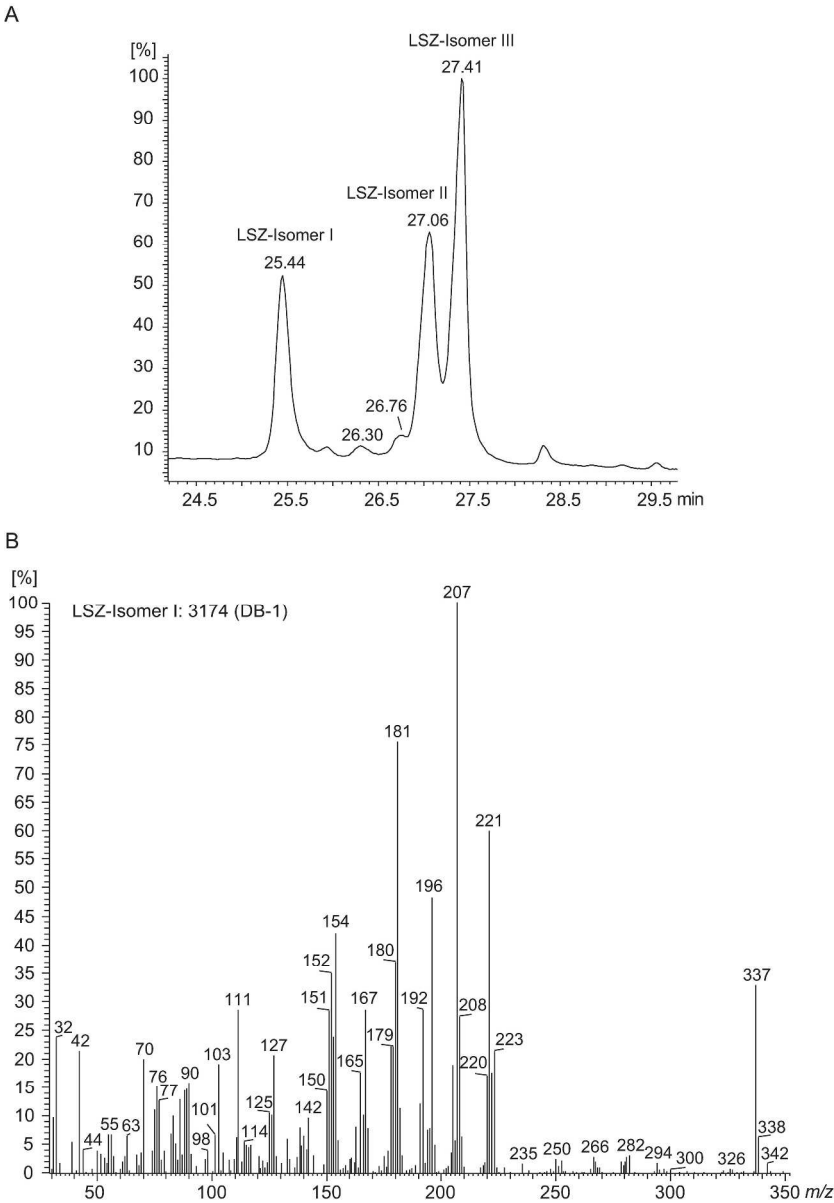


Figure 3. (A). Partial gas chromatography mass spectrometry trace where five LSZ isomers were detected. Three major isomers were labeled as LSZ I–III. Two minor isomers were also detected at 26.30 and 26.76 min. The corresponding mass spectra are shown as Supporting Information. (B). Electron ionization mass spectrum (EI-MS) recorded for LSZ isomer I.

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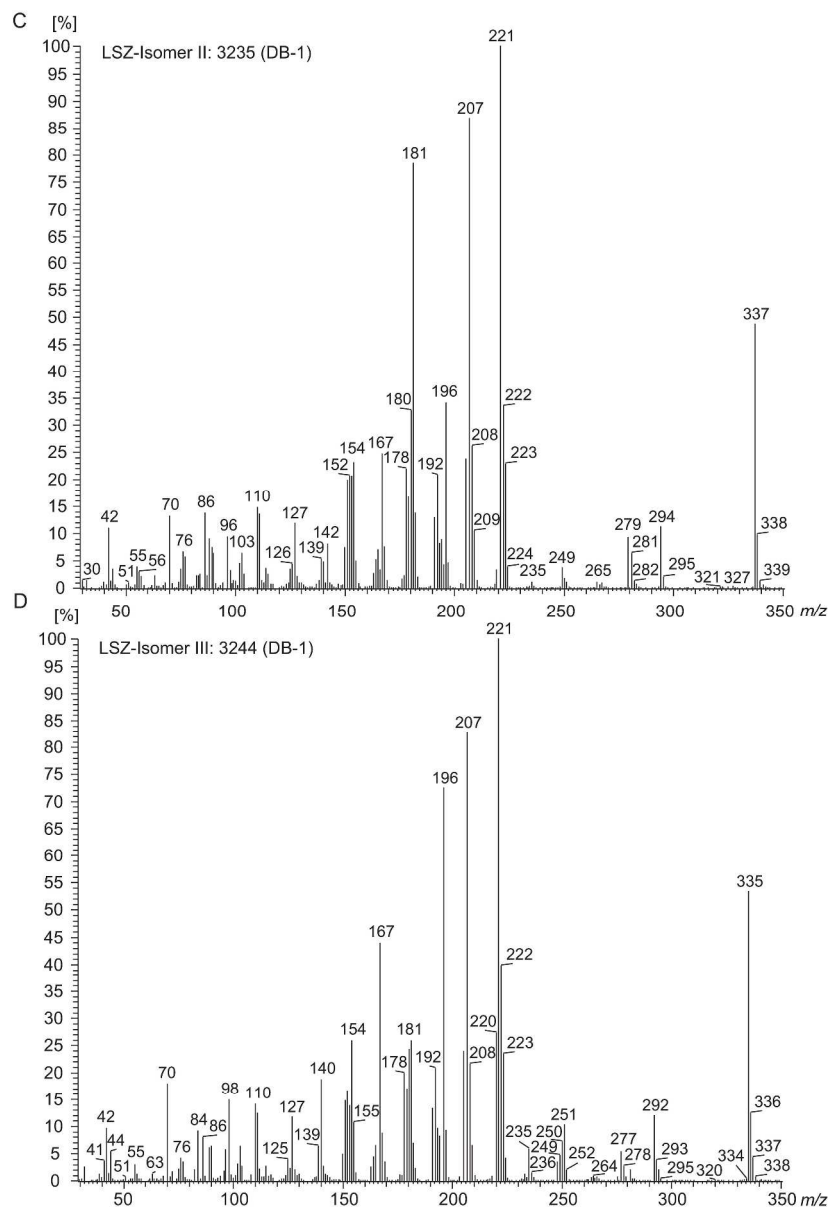


Figure 3. (C) and (D). Electron ionization mass spectra recorded for LSZ isomers II and III.  
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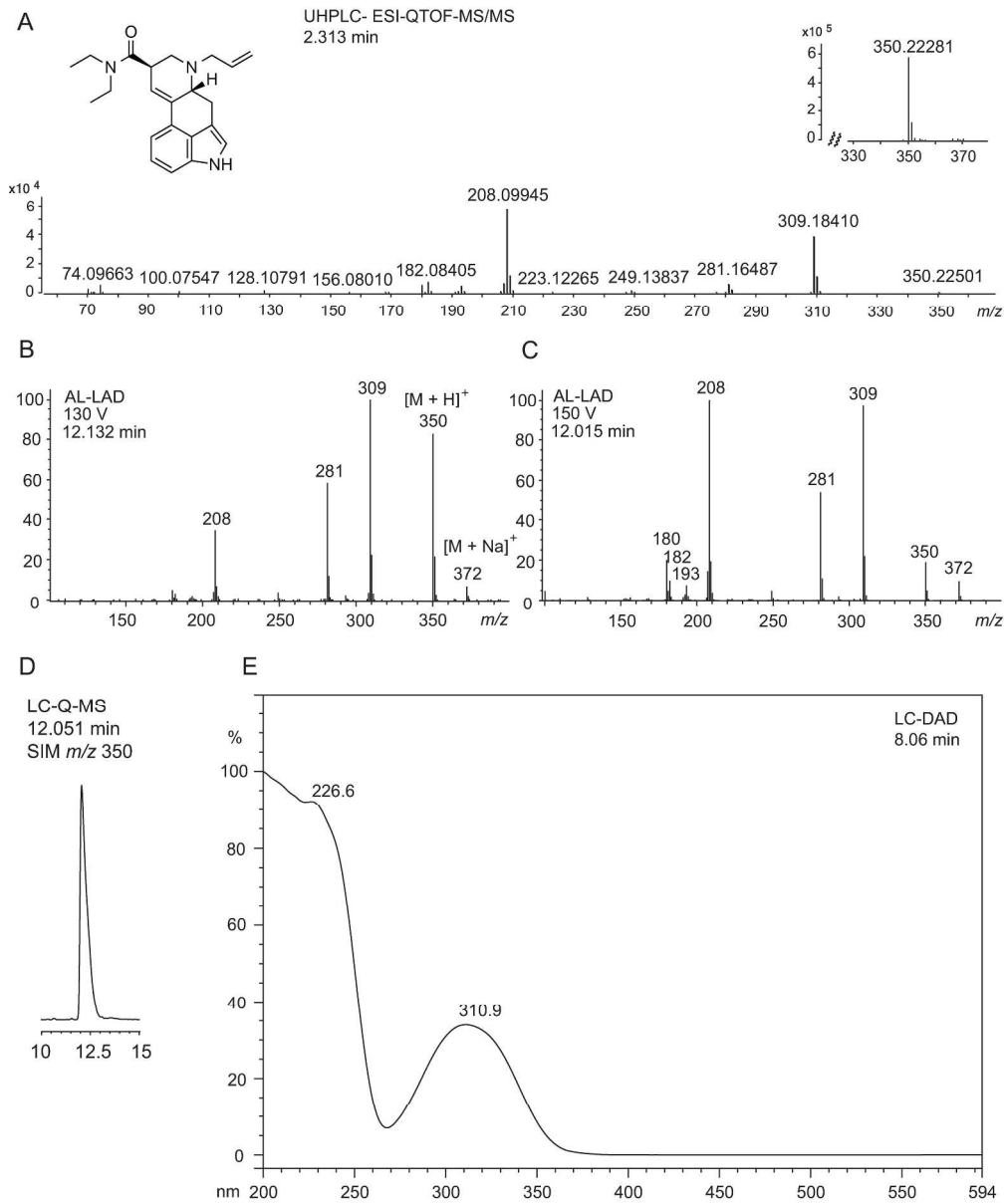


Figure 4. Comparison of electrospray ionization single and tandem mass spectra. (A). Quadrupole time of flight tandem mass spectrum (ESI-QTOF-MS/MS) recorded for AL-LAD. (B) and (C). In-source CID spectra of AL-LAD under single quadrupole mass spectrometry (HPLC-Q-MS) conditions. (D). HPLC-Q-MS trace using the m/z value of the protonated molecule for selected ion monitoring (SIM). (E). HPLC diode array analysis of AL-LAD.

245x294mm (300 x 300 DPI)



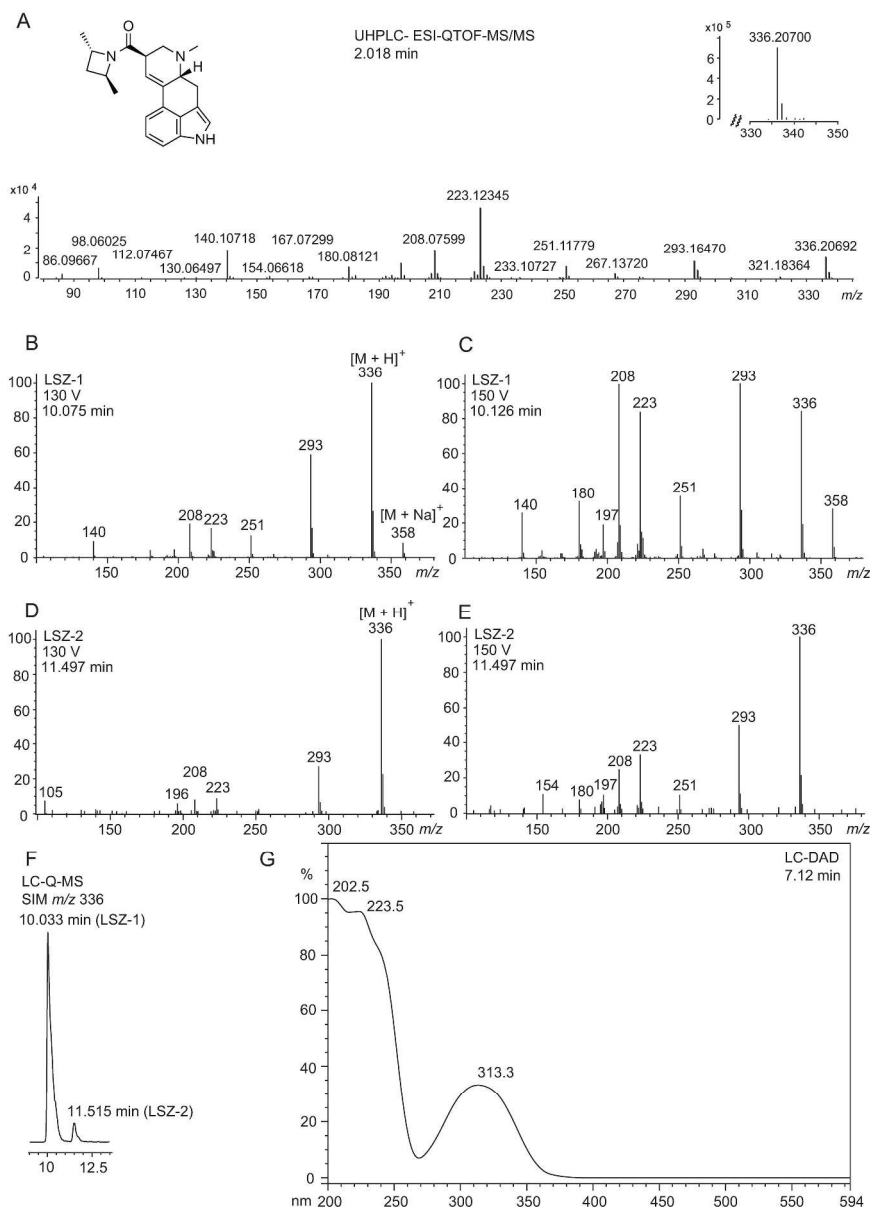


Figure 5. Comparison of electrospray ionization single and tandem mass spectra. (A). Quadrupole time of flight tandem mass spectrum (ESI-QTOF-MS/MS) recorded for LSZ. (B)/(C) and (D)/(E): In-source CID spectra of LSZ-I and LSZ-II under single quadrupole mass spectrometry (HPLC-Q-MS) conditions. (F). HPLC-Q-MS trace using the  $m/z$  value of the protonated molecule for selected ion monitoring (SIM). (G). HPLC diode array analysis of LSZ.

288x401mm (300 x 300 DPI)

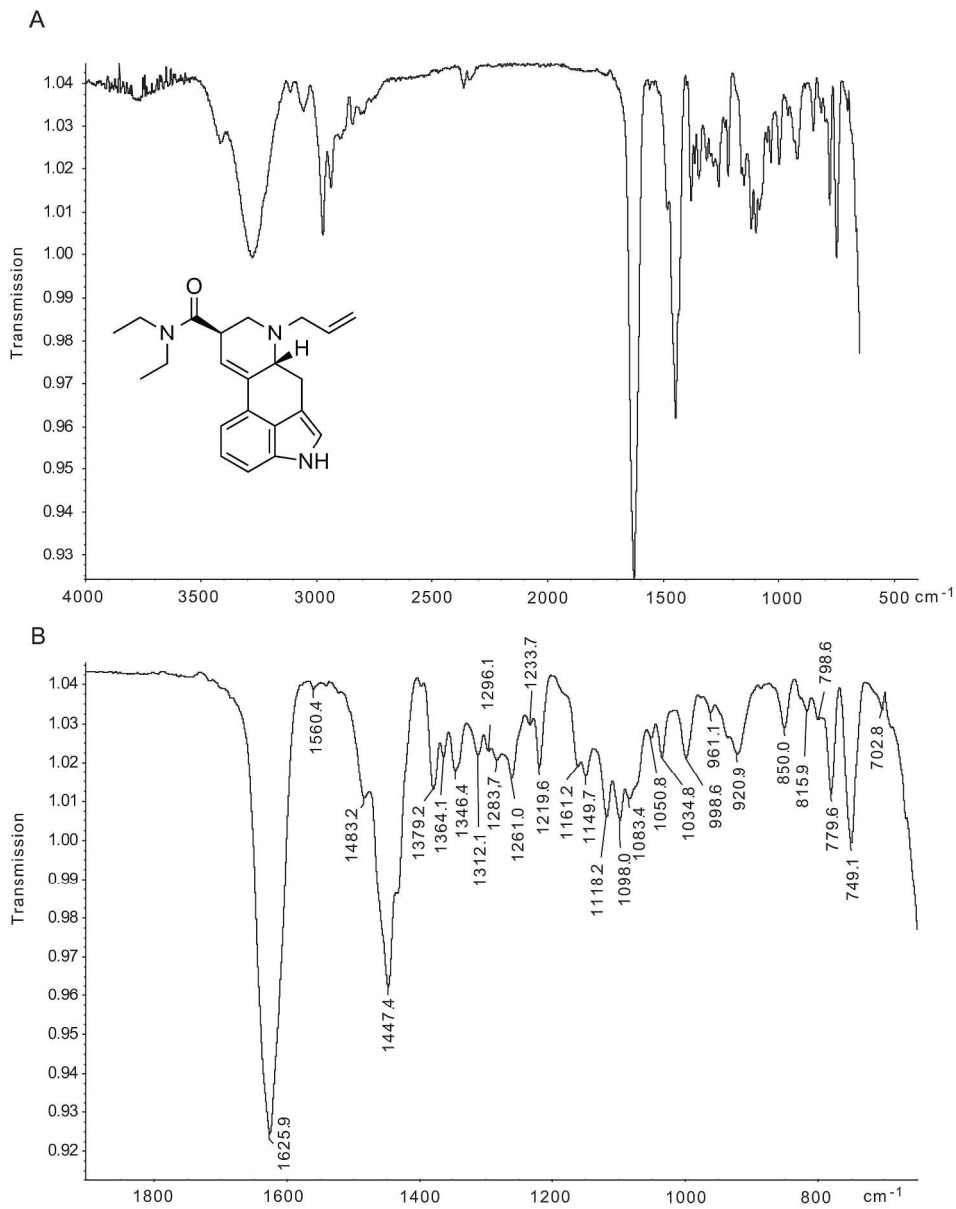


Figure 6. GC-solid state-IR spectrum of AL-LAD. Top: entire scan range. Bottom: partial scan range. 254x322mm (300 x 300 DPI)

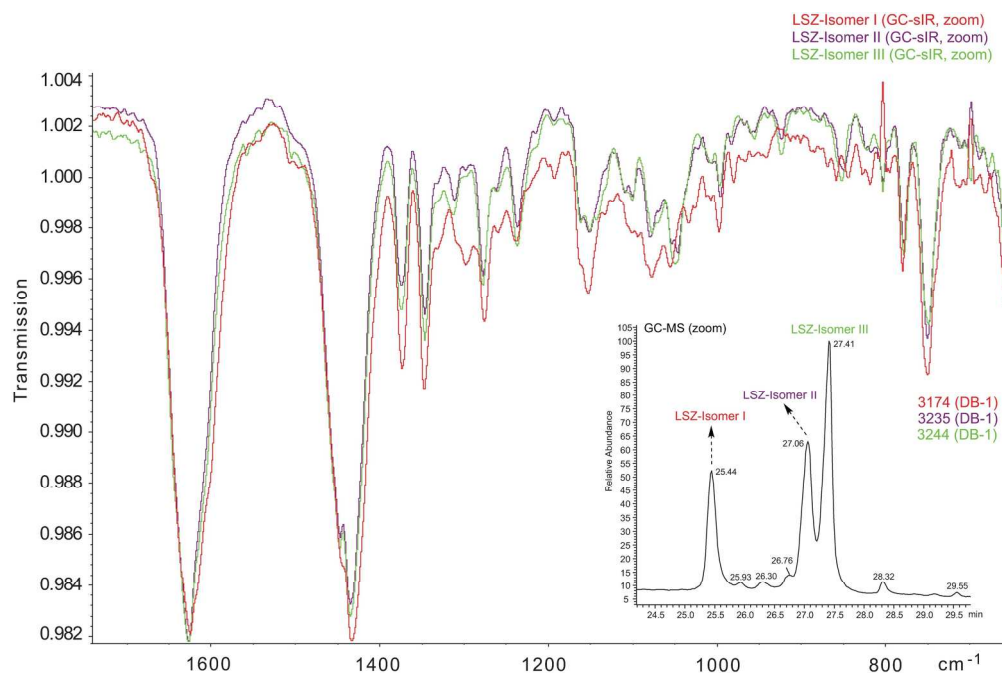


Figure 7. Partial, overlaid GC-solid state-IR spectra of LSZ isomers I–III for comparison. Individual spectra including the entire scan range are available as Supporting Information.  
191x127mm (300 x 300 DPI)

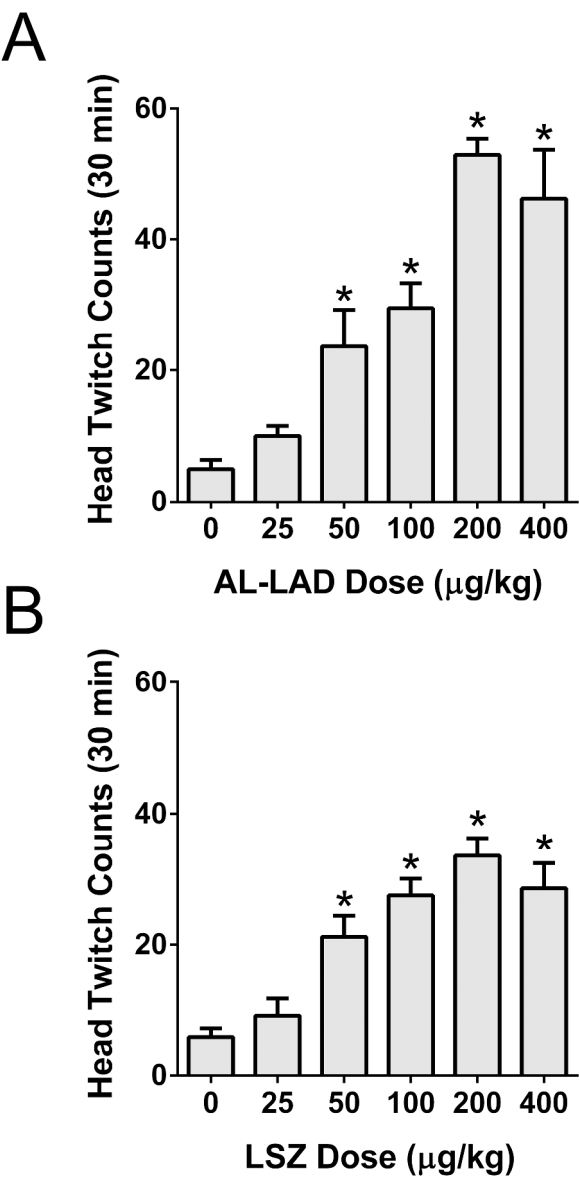


Figure 8. Effects of AL-LAD (A) and LSZ (B) on the head twitch response. Data are presented as group means  $\pm$  SEM for the entire 30-min test session. \* $p < 0.01$ , significant differences from vehicle control group (Tukey's test).  
219x440mm (300 x 300 DPI)

**Table 1.** Semi-quantitative estimation of lysergamides on blotter samples <sup>a</sup>

AL-LAD Control blotter	Added	Detected	Recovery [%]	AL-LAD blotter <sup>b</sup>	Amount (± SD)	Adjusted <sup>c</sup>
1	99.84 µg	90.46 µg	90.6	1	143.03 ± 1.36 µg	156.32 µg
2	99.84 µg	92.97 µg	93.1	2	144.24 ± 1.36 µg	157.64 µg
3	99.84 µg	90.69 µg	90.8	3	168.31 ± 1.47 µg	183.95 µg
Mean	99.84 µg	91.37 µg	91.5	4	198.00 ± 1.61 µg	216.39 µg
LSZ Control blotter				LSZ blotter <sup>d</sup>	Amount (± SD)	Adjusted <sup>e</sup>
1	114.30 µg	107.32 µg	93.9	1	113.58 ± 2.03 µg	121.74 µg
2	114.30 µg	105.86 µg	92.6	2	121.22 ± 2.09 µg	129.92 µg
Mean	114.30 µg	106.59 µg	93.3	3	121.89 ± 2.10 µg	130.64 µg
				4	104.11 ± 1.94 µg	111.59 µg

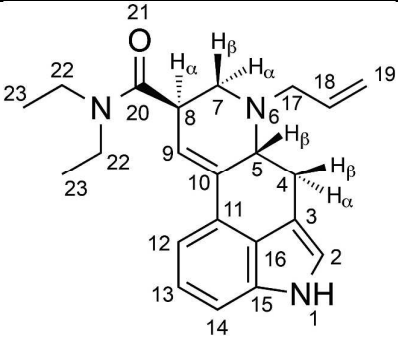
<sup>a</sup> LSD was used as internal standard whereas synthesized (2'S,4'S)-LSZ and powdered AL-LAD material were used as standards. AL-LAD:  $r^2 = 0.9999$ ; slope ± SD:  $0.02384 \pm 0.00011$ ; intercept ± SD:  $0.004005 \pm 0.016780$ . LSZ:  $r^2 = 0.9997$ ; slope ± SD:  $0.02778 \pm 0.00024$ ; intercept ± SD:  $0.06352 \pm 0.02953$ .

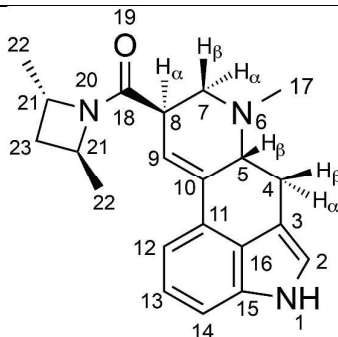
<sup>b</sup> Based on AL-LAD hemitartrate (see NMR data).

<sup>c</sup> Based on 91.5% recovery from control blotters.

<sup>d</sup> Based on freebase equivalents (see NMR data).

<sup>e</sup> Based on 93.3% recovery from control blotters.

<b>Table 2.</b> <sup>1</sup> H and <sup>13</sup> C NMR data for AL-LAD hemitartrate in d <sub>6</sub> -DMSO at 700 / 176 MHz		
		
No.	<sup>13</sup> C [δ / ppm]	<sup>1</sup> H [δ / ppm]
1	–	10.70 (d, <i>J</i> = 2.0 Hz, 1H)
2	119.31	7.02 (t, <i>J</i> = 2.0 Hz, 1H)
3	108.82	–
4	26.60	3.53 (dd, <i>J</i> = 14.5, 5.4 Hz, 4β-H, 1H) 2.55 – 2.46 (d, <i>J</i> = 11.8 Hz, 4α-H, 1H) <sup>a</sup>
5	59.71	3.38 – 3.34 (m, 5β-H, 1H)
6	–	–
7	51.72	3.08 (dd, <i>J</i> = 11.1, 4.5 Hz, 7α-H, 1H) 2.62 (t, <i>J</i> = 10.7 Hz, 1H), 7β-H, 1H)
8	39.05	3.77 – 3.71 (m, 8α-H, 1H)
9	120.31	6.23 (s, 1H)
10	135.56	–
11	127.47	–
12	111.03	7.01 (d, <i>J</i> = 7.02, 1H)
13	122.28	7.05 (t, <i>J</i> = 7.6 Hz, 1H)
14	109.83	7.18 (d, <i>J</i> = 7.9 Hz, 1H)
15	133.78	–
16	125.84	–
17	56.35	3.66 (dd, <i>J</i> = 14.5, 4.8 Hz, 1H) 3.18 (dd, <i>J</i> = 14.5, 8.0 Hz, 1H)
18	134.68	5.97 (dddd, <i>J</i> = 17.7, 10.2, 8.0, 5.0 Hz, 1H)
19	118.04	5.30 (d, <i>J</i> = 17.1 Hz, 1H) 5.20 (d, <i>J</i> = 10.2 Hz, 1H)
20	170.68	–
21	–	–
22	41.50	3.46 (dq, <i>J</i> = 14.4, 7.2 Hz, 1H) 3.40 (dq, <i>J</i> = 14.4, 7.2 Hz, 1H)
22	39.48	3.31 (q, <i>J</i> = 7.1 Hz, 2H)
23	14.81	1.17 (t, <i>J</i> = 7.0 Hz, 3H)
23	13.07	1.06 (t, <i>J</i> = 7.0 Hz, 3H)
TA <sup>b</sup>	72.03	4.26 (s, 1H)
TA <sup>b</sup>	173.20	–
<sup>a</sup> Overlapping with solvent		
<sup>b</sup> TA: tartaric acid		

**Table 3.**  $^1\text{H}$  and  $^{13}\text{C}$  NMR data for LSZ tartrate in  $\text{d}_6\text{-DMSO}$  at 700 / 176 MHz

No.	$^{13}\text{C}$ [ $\delta$ / ppm]	$^1\text{H}$ [ $\delta$ / ppm]
1	—	10.73 (d, $J$ = 1.6 Hz, 1H)
2	119.40	7.04 (t, $J$ = 1.9 Hz, 1H) <sup>a</sup>
3	108.44	—
4	26.39	3.51 (dd, $J$ = 14.5, 5.6 Hz, 4 $\beta$ -H, 1H) 2.56 – 2.51 (m, 4 $\alpha$ -H, 1H) <sup>b</sup>
5	62.41	3.21 – 3.16 (m, 5 $\beta$ -H, 1H)
6	—	—
7	54.81	3.08 (dd, $J$ = 11.0, 5.0 Hz, 7 $\alpha$ -H, 1H) 2.67 (t, $J$ = 10.8 Hz, 7 $\beta$ -H, 1H)
8	39.03	3.48 – 3.44 (m, 8 $\alpha$ -H, 1H)
9	119.23	6.25 (s, 1H)
10	135.22	—
11	126.85	—
12	111.12	7.05 (d, $J$ = 7.2 Hz, 1H) <sup>c</sup>
13	122.33	7.07 (t, $J$ = 7.2 Hz, 1H) <sup>d</sup>
14	109.96	7.20 (dd, $J$ = 7.2, 1.3 Hz, 1H)
15	133.83	—
16	125.72	—
17	42.98	2.54 (s, 3H) <sup>e</sup>
18	170.71	—
19	—	—
20	—	—
21	55.74	4.66 – 4.59 (m, 1H)
21	53.52	4.39 – 4.31 (m, 1H)
22	22.51	1.44 (d, $J$ = 6.2 Hz, 1H)
22	20.10	1.35 (d, $J$ = 6.3 Hz, 1H)
23	31.26	1.99 (ddd, $J$ = 11.1, 8.2, 5.8 Hz, 1H) 1.95 (ddd, $J$ = 11.1, 8.1, 5.1 Hz, 1H)
TA <sup>f</sup>	71.98	4.22 (s, ~1.6H)
TA <sup>f</sup>	173.37	—

<sup>a</sup> Overlapping with H-12 and H-13.<sup>b</sup> Overlapping with 17-CH<sub>3</sub>.<sup>c</sup> Overlapping with H-2 and H-13.<sup>d</sup> Overlapping with H-2 and H-12.<sup>e</sup> Overlapping with 4 $\alpha$ -H<sup>f</sup> TA: tartaric acid

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**Table 4.** Results of the head twitch response (HTR) studies

Agent	N	Dose (µg/kg)	HTR Counts (± SEM) <sup>a</sup>	ED <sub>50</sub> (µg/kg) <sup>b</sup>	ED <sub>50</sub> (nmol/kg) <sup>b</sup>
LSD <sup>c</sup>	5	0	3.8 (0.6)		
	5	50	40.8 (5.1) *		
	5	100	75.4 (11.4) *		
	5	200	83.8 (5.9) *		
	5	400	58.6 (2.9) *	52.9 (38.9–72.0)	132.8 (97.6–180.7)
AL-LAD	5	0	5.0 (1.4)		
	5	25	10.0 (1.5)		
	5	50	23.6 (5.6) *		
	6	100	29.5 (3.8) *		
	6	200	53.0 (2.4) *		
	5	400	46.2 (7.6) *	74.2 (55.3–99.7)	174.9 (130.2–234.8)
LSZ <sup>d</sup>	5	0	5.8 (1.3)		
	5	25	9.2 (2.6)		
	5	50	21.2 (3.2) *		
	6	100	27.5 (2.6) *		
	5	200	33.6 (2.7) *		
	5	400	28.6 (3.9) *	52.0 (37.3–73.5)	114.2 (82.0–159.1)

<sup>a</sup> HTR was assessed for 30 min. \* $p < 0.01$  vs vehicle (Tukey's test)

<sup>b</sup> With 95% confidence limits.

<sup>c</sup> Previously reported by Halberstadt and Geyer.<sup>[35]</sup>

<sup>d</sup> The potency calculation (in nmol/kg) was adjusted based on the proton NMR with regards to the LSZ / tartaric acid integrals.